EPA United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	**Algal toxin deposition in mouse hair and muscle: Potential biomarker of exposure and indicator of bioaccumulation.**
LAPR Number:	**17-12-001**
Principal Investigator	**Exemption 6**
Author of this Document:	**Exemption 6/RTP/USEPA/US**
Date Originated:	**11/03/2014**
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Date Approved:	**01/13/2015**
Date Closed:	**12/13/2017**

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 RTP/USEPA/US by Exemption 6/RTP/USEPA/US	01/13/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	01/13/2015	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Algal toxin deposition in mouse hair and muscle: Potential biomarker of exposure and indicator of bioaccumulation.

Is this a continuing study with a previously approved LAPR?
Yes
Please provide the previous LAPR# 14-10-002

2. What is the Intramural Research Protocol (IRP) number covering this project?
IRP-NHEERL-TAD/DBB/[redacted]/2012-01-r01
SSWR 2.3c

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
[redacted]	[redacted] ***Lotus Notes Address*** [redacted]/RTP/USEPA/US	TAD ***Branch*** DTB	MD B105-04

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
[redacted]	[redacted] ***Lotus Notes Address*** [redacted]RTP/USEPA/US	TAD ***Branch*** DTB	MD B105-04

SECTION A - Description of Project

1. Study objectives, presented in <u>non-technical language</u> such that it is understandable by non-scientific persons, including how the study addresses health protection. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.
The potential of cyanobacterial toxin exposures in the environment to adversely affect the health of humans and

wildlife is an issue of increasing importance in keeping with the increased occurrence and severity of hazardous algal blooms (HABs) globally. The apparent increase in incidence, range, and severity of HABs in the United States and elsewhere is due to a variety of factors that differ across various localities. These include increased monitoring and awareness; climate change since warming of waters, both marine and freshwater can result in enlarged ranges and periods during which blooms can persist; increased transport of algal species, including toxin-producing types, globally; and increased nutrient loading (especially nitrogen and phosphorus) from agriculture, as well as other sources, in both marine and freshwater bodies. All of these factors, either alone or in combination, can result in the significantly increased presence of these superbly adapted and adaptable organisms. HABs can last for long periods of time, but more often are short-lived and dynamic phenomena. If data concerning a HAB's potential impact on human populations are not rapidly collected, the algal species and/or toxins of concern may have changed, and the data obtained by investigators will bear little relevance to conditions existing at the height of the bloom. This makes any attempts to relate human algal toxin exposures to subsequent adverse health outcomes extremely difficult or impossible. Adequate methods to directly measure the levels of toxins in exposed human populations are therefore needed.

Algal toxin exposures through drinking or recreational waters have justifiably received much attention, but the historical record clearly indicates that instances of algal poisoning through the ingestion of bioaccumulated toxins in foods have caused a majority of events of severe health effects in humans. This is reflected in the common names of many dangerous toxins: paralytic shellfish poisoning (saxitoxins), neurotoxic shellfish poisoning (brevetoxins), amnesic shellfish poisoning (domoic acid); ciguatera fish poisoning (ciguatoxin), and diarrhetic shellfish poisoning (okadaic acid). Harmful exposures to these toxins in mammals including humans, are only known to have occurred after the ingestion of vertebrates and invertebrates that have bioaccumulated (concentrated) these toxins. Documented poisoning episodes have, for example, been shown to be caused by saxitoxins in shellfish eaten by humans, microcystins in shellfish eaten by sea otters, and domoic acid and ciguatoxins in fish or shellfish eaten by birds, marine mammals and humans.

The potential bioaccumulation of algal toxins in the meat and associated fat that humans consume is, at present, unstudied. This possibility, however, is worthy of investigation since instances of algal poisoning of cattle have been documented in Kansas, Montana and Georgia and cattle have possibly been sent to market before they exhibited symptoms of the algal toxins. Knowledge of the potential bioaccumulation of a variety of toxins in fat and meat (i.e., muscle) may be of practical use in assessing possible health risks from herds of animals exposed to these chemicals in the water they drink.

The primary objectives of the research covered in the LAPR are:
1. To evaluate the potential of toxin deposition in hair to indicate the extent of exposures to a variety of algal toxins in animal and/or human populations. The potential importance of these studies for the evaluation of human exposures to toxins in hazardous algal blooms (HABs) is extremely significant. If the strategy being tested proves to be valid, it will enable regulatory agencies to evaluate past exposures and relate those exposures to adverse health effects, and also, therefore, increase the accuracy of the EPA's ability to estimate human exposures for threatened or existing HABs. This, in turn, will greatly improve the scientific rationale for decisions made on maximum allowable exposures in both recreational and drinking waters. Currently, assessments of human exposures to HABs are at best imprecise since even the simplest methods involve obtaining samples of blood or urine, which is logistically difficult or impossible to do immediately after, or during, the occurrence of the bloom. Delayed sampling is often of little use since the half-lives of most algal toxins in blood or urine are measured in hours or a few days. Society has faced the same issue concerning the need for human exposure data for a number of other chemicals including heavy metals such as lead or arsenic; performance enhancing steroids in sports; and illegal drugs such as heroin, cocaine, amphetamines and PCP (phencyclidine). There are generally three approaches to human drug testing that involve legally mandated samples taken from individuals: the use of blood, urine and hair. In situations involving HABs, however, both blood and urine analyses after algal blooms are generally extremely difficult to obtain in necessary numbers that allow for accurate conclusions. This is especially true for children who are often the most exposed demographic in recreational waters. Obtaining samples of hair for toxin analyses is both easier to carry out and, most importantly, the toxin should have a much slower turnover rate in hair due to the fact that the chemicals are deposited and essentially stored in the shafts. Most drug exposures in humans can be characterized for 3 months or more. This hair deposition testing strategy is used globally, and hair analyses to detect the agents listed above are routinely utilized in Europe and North America under standardized guidelines.

2. Fetal exposure in utero and levels of toxins in milk will be determined. This is a neglected area of toxicology although it may prove to be important in terms of human exposure. Our data with cylindrospermopsin (CYN) demonstrates that the late term fetus is more sensitive than the pregnant dam. This has medical implications for the new-born baby, since maternal exposure may produce adverse fetal effects at levels that do not produce noticeable effects in the pregnant female. Exposure via the milk is a mechanism that may expose a vulnerable sub-population to toxic levels of cyanotoxins, many of which are lipophilic (i.e., fat soluble, and may have higher concentrations in milk).
3. The potential for bioaccumulation of algal toxins in muscle and fat will be evaluated in the same animals undergoing testing for hair deposition. The basic experimental design should allow a determination of the levels of algal toxins in fat and muscle tissues during repeated exposures over a period of 1 1/2 months. This period of time will be sufficient to determine if tissue levels are increasing, and in the event this occurs, additional studies using longer exposure times may be used to fully assess bioaccumulation potential.

Except for our own efforts, the use of the hair analysis strategy to test for exposures to algal toxins has not been tested in the laboratory. In our previous LAPR we explored this, allowing us to develop an adequate strategy for obtaining repeated samples of hair from mice that should be sufficient to allow analyses for toxins that have been deposited in the hair shafts. Very preliminary work with microcystin-LR using enzyme-linked immunosorbent assay (ELISA) indicated its probable presence in hair samples. ELISA is not as sensitive as the methods to be used here (liquid chromatography/mass spectrometry), so these data may indicate the feasibility of this approach.

Bioaccumulation studies have been sporadically done in plants, invertebrates, and fish, but there have been no systematic efforts to evaluate algal toxin accumulation of a broad series of different toxins in mammalian fat and/or muscle - the two tissues most commonly eaten by human populations. The study proposed here will address this issue and will consist of standard algal toxin exposure studies in mice, extraction of organic compounds (including algal toxins) from hair and tissues, and subsequent analysis by validated analytical chemistry methodologies. The exposures and initial separation of toxins from tissues will be performed at the RTP. The final analyses of hair will be done by [redacted], U.S. Geological Survey, Organic Geochemistry Research Laboratory, Lawrence, Kansas. Analyses of tissue samples will be done by [redacted], National Oceanographic and Atmospheric Agency, Hollings Marine Laboratory, Charleston, South Carolina. [redacted] are analytical chemists who are experienced in the detection of algal toxins.

The proposed study will evaluate hair deposition as a possible quantitative biomarker of algal toxin exposure, and a survey of a series of algal toxins known to have adversely affected humans and wildlife. The current proposal is designed to test the toxins singly and if successful, will be followed by additional studies proposed in amendments to evaluate additional toxins and mixtures.
The current Proposal will use five toxins:
1. Microcystin-LR (M-LR) (a ubiquitous, potent hepatotoxin thought to be a human carcinogen and known to bioaccumulate in invertebrates)
2. Cylindrospermopsin (CYN) (a cytotoxin thought to have caused severe toxicity in humans after ingestion in drinking water, and now known to occur in many U.S. lakes)
3. Anatoxin-a (A-a) (a potent neurotoxin thought to be responsible for human, livestock, and pet fatalities in several states during the last 3 years
4. Rubidium chloride (Rb) (an alkali metal salt that has been associated with algae in areas where toxic algal blooms are known to have occurred)
5. Saxitoxin (Stx) (paralytic shellfish poison, marine and freshwater neurotoxin known to have caused deaths in humans who consumed invertebrates in which bioaccumulation occurred).

The exposure of perinatal animals to algal toxins has received virtually no attention. The developing embryo, fetus, and pre-weanling animals constitute a potentially susceptible population of pre-pubertal animals. There are three basic routes of exposure in these populations: placental, lactational, and dietary. The extrapolation of toxin exposure effects in adults may not be applicable to pre-pubertal populations because of differences in exposure routes, the maturity of metabolic processes, and ongoing developmental processes that may be uniquely susceptible to disruption. In utero developmental toxicity is assessed by standardized bioassays but the fetal and pre-weaning responses are not covered in these bioassays and most often go unstudied. The studies proposed here will form a broad assessment of the extent of fetal and perinatal exposure to a series of the most common freshwater algal toxins. Significant levels of toxins in the newborns or milk may point to other routes of exposure that may need to be considered during the generation of exposure guidelines.

The ability of algal toxins to accumulate in muscle tissue is of potential importance for safety of the meats consumed by our population. As stated earlier, a great deal of human toxicity resulting from algal toxins has been a result of exposure through meats from animals that have bioaccumulated significant amounts of toxins. Much of the work on bioaccumulation has centered on marine toxins, and freshwater toxins have been relatively unstudied. The studies detailed in this Proposal will form an initial survey of this potential route of exposure for commonly found freshwater toxins.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

Medline was searched from 1980 to present and we could find no validated in vitro methods or computer model systems that can substitute for laboratory animals in a study of this nature. This is related to the fact that the study endpoints involve the transport and deposition of the toxins in the shaft of the hair and accumulation in muscle and fat. These endpoints can only be tested in an in vivo system since long-term studies are not possible in in vitro systems and the necessary pharmacokinetic/metabolism information needed for effective computer programs are not well-known for these compounds.

b. Justify the species requested:

The mouse is a commonly used laboratory animal that is readily available. Most of the work with cyanotoxins has been done with the mouse because of the difficulty in obtaining sufficient quantities of the toxins for use with larger laboratory animals such as the rat.

3. How was it determined that this study is not unnecessary duplication?

A search of the literature from 1970 to the present on PubMed using the search terms, "hair" and "deposition" "cyanotoxins", "algal toxins" and "bioaccumulation" failed to locate any relevant in vivo studies with mammals.

SECTION B - In Vivo Procedures

1. Briefly describe experimental design. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

We will use pregnant and non-pregnant cohorts to study both hair deposition and bioaccumulation in muscle and fat.

Please note: A flow chart summarizing both the non-pregnant and pregnant animal studies is attached to the LAPR.

Animals used will be young post-pubertal male and female C57BL6 mice weighing approximately 25g, and pregnant animals. (This strain offers an advantage as these toxins tend to accumulate more readily in pigmented hair. However, if we have low pregnancy rates with this strain, as has happened in the past, we will use CD-1 mice for the pregnant cohorts.)

Each toxin experiment (both non-pregnant and pregnant animals) will involve a total of 120 animals (30 males and 30 female non-pregnant and 60 pregnant). All studies will consist of 42 toxin-exposed and 18 controls.

The chemicals will be administered i.p. over three discrete dosing periods (5 days, 3 days, and 3 days) over a 32-day period (see attached flow chart). Rubidium will be dosed by gavage for 32 consecutive days. Controls will receive similar volumes of vehicle: physiological saline i.p., or deionized water by gavage.

The intermittent dosing schedule allows for some degree of recovery. There are no published data on extended treatments of these compounds by the i.p. route that we can use as guides. We are attempting to expose animals to a large cumulative dose that does not cause severe toxicity and this appears the safest strategy to accomplish this goal. Humans generally get exposed to algal toxins intermittently (like the HABs) over periods of weeks/months. The strategy of the protocol is to expose animals to maximum non-toxic or mildly toxic levels over a period of time long enough to evaluate the possibility of bioaccumulation. Period of time between last dose and tissue collection: we do not know what the growth rate of mouse hair is and have to assume that the last dose will be deposited in the shafts during a 1-2 week period of time. If it is obvious that more or less time will be optimal, we will submit an amendment to change the protocol to reflect what we have found.

There will be three points during the study when animals are euthanized. The euthanasia schedule (shown in flow chart) is the same for all five chemicals.

Non-pregnant cohort.
Sixty animals (30 males, 30 females) will be dosed for 5 consecutive days (Days 1-5). Twenty of these animals (10 males and 10 females) will be euthanized 7 days after the initial dosing period on Day 12. On Days 13-15, the remainder of the animals (40) will be dosed. On Day 29, 20 animals (10 males and 10 females) will be euthanized. On Days 30-32, the remaining 20 animals (10 males and 10 females) will be dosed for 3 consecutive days. On Day 46, the final 20 animals will be euthanized. Each euthanasia time point will have 10 males and 10 females, of which 7 of each sex will be treated with toxin and 3 will be controls.

Pregnant cohort.
Sixty timed-pregnant females will be dosed on gestational days (GD) 13-17 (Day 1-5). Twenty of these animals will be euthanized on GD18/PND1 (Day 6). To allow collection of both fetal and pup tissues, approximately 10 dams will be euthanized before parturition (GD 18), and approximately 10 dams and litters will be euthanized after parturition [postnatal day (PND) 1]. Fetal tissue levels will be used to evaluate potential placental transfer of the toxins. Milk will be obtained from the stomachs of the PND1 pups and analyzed as a measure of potential transfer of toxins during lactation. On PND 1 (Day 6), remaining litters will be normalized to 10 pups (5 males, 5 females, if possible). Dams will receive their second round of dosing for 3 days on PND 8-10 (Days 13-15). On PND 20-21 (Days 25, 26), litters will be weaned. Most weanlings will be euthanized; selected weanlings will be maintained for tissue sampling at up to PND 41. Pup growth and viability will be evaluated throughout the course of the study. Twenty dams will be euthanized at PND 24 (Day 29). The remaining 20 dams will be dosed on PND 25-27 (Days 30-32) and euthanized on PND 41 (Day 46). Each euthanasia time point will have14 dams exposed to toxin and 6 control dams.

During the course of the study, animals will be weighed daily as well as examined for clinical signs of toxicity. At euthanasia, all adult animals will be weighed and appearance of tissues recorded. Blood will be obtained for clinical chemistries and toxin analysis. Hair, liver, fat (from abdominal fascia), and muscle (from thighs of rear legs) will be collected for chemical analysis of toxin levels and histopathology (if feasible). Brain tissue will also be collected from anatoxin-a and saxitoxin-treated animals as both of these compounds are neurotoxins that directly affect the functioning of the brain.

Hair samples will be removed (using an Oster Trimmer Hair Clipper) from all animals at each euthanasia. Analysis of hair will constitute a test for using this tissue as a means of assessing exposure. Toxin levels in hair may be compared to levels in blood and liver tissue. Toxins in fetal and pup tissues will be compared to levels in the dams, and this will be done on a per litter basis so that inter-litter variability can be assessed.

Hair and other tissues will undergo a methanol/water extraction using a protocol supplied by Exemption 6 and it is anticipated that this will hasten the LC/MS analyses done by others. The hair extracts will be shipped to the USGS Laboratory, and tissue extracts will be shipped to Dr. Peter Moeller (NOAA, Hollings Marine Laboratory) for analysis.

Section D lists the cyanotoxins to be used; the dose levels to be employed, the expected effects, and the references and/or rationales used for the dose selection. We have attempted to use doses that are at or near "effect levels" without inducing morbidity since the primary questions deals with hair and tissue depositions. Such dose levels will enable us to draw sound conclusions as the levels of toxins will be meaningful to potential exposures leading to adverse health effects. The i.p. route will be used in this portion of the project for all cyanotoxins since it uses significantly less toxin than the oral (where known, the oral route is at least 10X less effective than the i.p. route), whereas the oral route can be used for rubidium as it is much more easily available for use in experimental studies. The endpoints of these studies include an evaluation of the potential deposition and subsequent identification of these toxins by chemical analysis, the transfer of toxins to offspring across the placenta or via milk, and the ability of the toxins to bioaccumulate in muscle and fat.

NOTE: We do not anticipate severe toxicity with the dosing regimens we will be using; however, because of the potential for unexpected adverse health effects, all animals exposed to toxin will be considered Category E.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

A critical component of these studies involves analytical chemistry. Both [redacted] recommend a minimum of 10-20 animals per compound for each time point. Based on the anticipated individual variability, each toxin study is designed to use 60 non-pregnant (30 males, 30 females; 42 exposed to toxins and 18 controls receiving vehicle) and 60 pregnant animals (42 treated and 18 controls). We are requesting the 60 animals to allow for animals assumed to be pregnant that are not. This is a total of 300 non-pregnant mice (150 of each sex) of which 210 will be toxin-exposed and 90 will be controls). A total of 300 pregnant animals will be used, comprising 210 toxin-exposed and 90 controls. This will require a total of 300 non-pregnant and 300 pregnant animals to test the five cyanotoxins. It will also result in in the range of 1750 pups from treated, and 750 from control dams allowed to give birth (assuming an average litter size of 10).

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): <u>Please enter numbers only.</u>

Categories	***Adults***	***Offspring***
C) Minimal, transient, or no pain/distress:	180	750
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:	420	1750

4. For tracking purposes, please check if this LAPR includes any of the following:

- ☐ Restraint (>15 Minutes)
- ☐ Survival surgery
- ☐ Food and/or water restriction (>6 Hours)
- ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

Control animals be dosed for 5 consecutive days; 7 days later with 3 consecutive doses; and 2 weeks later, with 3 consecutive doses for each toxin study. Sterile physiological saline will be administered by the i.p. route using a 26g needle. All volumes will be 0.1ml/dose.

Controls for rubidium chloride will be dosed by gavage with 0.2ml/day of deionized water for 32 consecutive days.

b. Survival Blood Collections (method, volume, frequency):

none

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

none

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

none

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

f. Describe how animals will be monitored (e.g., frequency of observations, by whom):

Daily (including weekends and holidays) by [redacted] with the assistance of [redacted] At term, dams will be monitored for parturition and dystocia. Dams exhibiting dystocia will be euthanized.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

Microcystin-LR, anatoxin-a, cylindrospermopsin, and saxitoxin will all be dosed for 5 consecutive days; 7 days later with 3 consecutive doses; and 2 weeks later, with 3 consecutive doses. These compounds will be administered by the i.p. route using a 26g needle. All volumes will be 0.1ml/dose and the toxins will be administered in sterile physiological saline. The doses used will be: Microcystin-LR - 30ug/kg/day; Cylindrospermopsin - 40ug/kg/day for non-pregant animals and 50ug/kg/day for pregnant animals; anatoxin-a - 200ug/kg/day; and saxitoxin - 6ug/kg/day.
Rubidium chloride will be dosed with 18mg/kg/day for 30 consecutive days. Dosing will be by gavage and the compound will be delivered in 0.2ml of deionized water.

b. Survival Blood Collection (method, volume, frequency):
none

c. Testing methods:
none

d. Restrictions placed on the animals' basic needs (e.g., food and/or water deprivation, light cycles). Provide details regarding the length of deprivation:
none

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):
Daily (including weekends and holidays) by Exemption 6 with the assistance of Exemption 6 Exemption 6. At term, dams will be monitored for parturition and dystocia. Dams exhibiting dystocia will be euthanized.

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:
none

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:
No treatment-related deaths are expected.

7. Surgical Category D and E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:
none

b. Anesthetic regimen (drugs, dosages, volume, and route of administration). The use of paralytic or neuromuscular blocking agents without anesthesia is prohibited:
none

c. Postoperative care (thermal support, special feeding, frequency and duration of monitoring, responsible personnel, removal of sutures/staples):
none

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):
none

e. Will any animals be subject to more than one major surgical survival procedures?
○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:
none

8. Humane interventions (for treatments/procedures in all categories).

a. Describe actions to be taken in the event of expected or unexpected deleterious effects from procedures or chemical exposures.
In the event of unexpected toxicity, the Attending Veterinarian will be consulted regarding disposition of the animals.

b. State criteria for determining temporary or permanent removal of animals from the study.
Animals losing weight, but still active and eating, will be monitored at an increased frequency. If we observe animals exhibiting significantly declining condition, we will euthanize them. Dams exhibiting dystocia will be euthanized. Toxicity warranting euthanasia will include changes in appearance (hunching and/or piloerection), loss of appetite and/or lack of fluid consumption, lethargy, and non-responsiveness to interaction. Necropsies will be performed and blood and tissues will be collected for analysis as needed.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

Analgesics will not be used because the interaction of analgesics with these compounds is unknown. The necessary study comparing treated animals with and without analgesics would actually lead to the use and eventual euthanasia of another set of animals. Also, the response to toxicity involves initiation of the stress cascade that, in itself, alters numerous normal responses to xenobiotics - the absence of the stress response would therefore create a situation that would make extrapolation to human or other animal populations more difficult. A PubMed search encompassing 1970 to the present, using the search terms analgesia and the names of the individual toxins in this study, failed to find any in vitro studies that investigated hair deposition or bioaccumulation.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study:	600
b. Animals to be transferred from another LAPR:	0
LAPR Number that is the source of this transfer:	
c. Animals to be transferred from another source:	0
d. Offspring produced onsite (used for data collection and/or weaned):	2500
e. TOTAL NUMBER of animals for duration of the LAPR	3100

2. Species (limited to one per LAPR): Mouse/Mice

3. Strain: C57BL/6 mouse/mice, CD-1 (for pregnant cohort if low pregnancy rate with C57BL/6)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

none

4. Sources of animals:

Charles River Laboratories

5. Provide room numbers where various procedures will be performed on animals:

Animal facility animal rooms [redacted].

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

no *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

none

8. Describe any unusual housing or husbandry requirements, or acclimation requirements.

Justify any treatment beginning less than 3 days after arrival.
none

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)
none

10. Housing and Enrichment.
The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.
Non-pregnant cohorts will be housed 3-4/cage for the duration of the study. Pregnant dams will be housed 3-4/cage prior to GD13, and then housed individually to allow litter identification. Weanlings will be segregated by sex and housed 3-5/cage. After weaning of their litters, dams will be housed 3-4 per cage. Polycarbonate cages with solid bottom, automatic water delivery, and pine shavings as bedding will be used. Enrichment will be limited to plastic tubes and igloos.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

The HSRP for the algal toxins is "HSRP-164".

Microcystin-LR (M-LR) 30ug/kg
Rao and Bhattacharya (1996) found an LD50 of 43ug/kg. Reducing the dose to 21ug/kg resulted in DNA strand breaks in the liver but no lethality. [redacted] et al. (2002), found histopathology at 32ug/kg given for two consecutive days, but no lethality.

Cylindrospermopsin (CYN) 50 ug/kg (pregnant), 40 ug/kg (non-pregnant)
In our previous studies, 50ug/kg/day did not produce overt toxicity in pregnant females and this is the dose we will use for this group. This dose regimen does produce overt toxicity in non-pregnant animals. We have assumed that this difference, coupled with the adult type of CYN toxicity seen in the neonates, indicates that the toxin is distributed throughout the dam, including the fetal compartment - toxicity, as a result, being lessened in the dam and present in the neonate. Excluding the calculated fetal compartment weight from the dam weight, our data indicate that the average levels of CYN in the dam (only) tissues averages 40ug/kg/day during GD13-17 and this is the dose that we will use for the non-pregnant animals [redacted] LD50 is 1100 ug/kg.

Anatoxin-a (A-a) 200ug/kg
We found that animals dosed with 200ug/kg exhibited reduced activity shortly after dosing, with recovery 15-20 minutes post dosing. [redacted]
LD50 in mice is unknown; oral dosing of 1125ug/kg for 10 days in hamsters was not lethal

Rubidium chloride 18mg/kg
Oral LD50 in mouse is 3800 mg/kg.
Minor weight loss and possible signs of neurotoxicity have been reported after dosing with 18mg/kg/day in the diet for 3 weeks. Follis, 1942.

Saxitoxin 6ug/kg
The LD50 of saxitoxin is >10ug/kg. Chronic studies in mammals have not been done using this route. Wiberg and Stephensen, 1960.

2. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in this LAPR, and provide:

a. Information to assure that such material is pathogen-free
none

b. A statement regarding any safety precautions necessary for handling the material.
none

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	*ROLE*	*SPECIFIC RESPONSIBILITY*	*RELEVANT TRAINING*
[redacted]	Principal Investigator	Design of study, dosing, participation in necropsy and tissue collection.	40 years of experience in animal toxicology studies. All relevant animal use NHEERL training courses.
[redacted]	Associate Principal Investigator	Design of study, participation in dosing, weighing, monitoring and necropsy, blood collection and coagulation analysis.	Licensed veterinarian. All relevant animal use NHEERL training courses.
[redacted]	Post-Doc	Participation in dosing, weighing, monitoring and necropsy, blood collection and coagulation analysis.	All relevant animal use NHEERL training courses.
[redacted]	Student	Participation in dosing, weighing, monitoring and necropsy, blood coagulation analysis.	All relevant animal use NHEERL training courses.

RTP-NHEERL	Tech Support	Category C Procedures	EPA IACUC Trained

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year	None
2. Breeding protocols and recordkeeping	None
3. Methods for monitoring genetic stability	None
4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR	None

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Adults will be euthanized on Study Day 46.
Offspring will be euthanized on PND 1, weaning, and postweaning up to PND 41.

2. Describe the euthanasia techniques:

Method(s):	Anesthesia plus exsanguination (adults & weanlings) Decapitation (fetuses/neonates)
Agent(s):	CO_2
Dose (mg/kg):	To effect
Volume:	Full
Route:	Inhalation

Source(s) of information used to select the above agents/methods:

_ Personal Experience, Common Agents for Anesthesia & Euthanasia
Extra scissors will be available for decapitation.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the 2007 American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

none

4. Describe how death is to be confirmed.

Vital organ section, Prolonged absence of breathing

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

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The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. The proposed research using animals does not unnecessarilty duplicate any previous experimentation.
4. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
5. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. Individuals from outside of EPA who are collaborating on this project, and who conduct related experimentation on EPA procured or bred animals in their respective Institutions, have the equivalent of a current IACUC approved LAPR at their respective Institutions.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	11/26/2014

Submitted: 11/26/2014

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	12/02/2014 by Exemption 6 Exemption 6 Exemption Exemption RTP/USEPA/US	Exemption 6 *Lotus Notes Address* Exemption 6 Exemption 6 Exemption Exemption RTP/USEPA/US	TAD *Branch* DTB	MD *Submitted to Branch Chief for Approval* 11/26/2014 02:15 PM

ATTACHMENTS

17-12-001 PI resp 1-7-15.pdf LAPR Flow Chart 17-12-001 Correction 12-19-14.pptx[attachment "LAPR17-12-001 Flowchart - Biomarker-Bioaccumulation Study.pptx" Exemption 6Exemption 6]

Actions

First Update notification sent: 11/04/2015
Second Update notification sent: 12/09/2016

First 2nd Annual notification sent: 11/02/2016
Second 2nd Annual notification sent:

1st Expiration notification sent: 10/30/2017
2nd Expiration notification sent: 12/04/2017

History Log: